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(54) Title: METHODS AND COMPOSITIONS FOR MULTIPLEX AMPLIFICATION OF NUCLEIC ACIDS

(57) Abstract

A method is described for predetermining ratios of primer pairs present in a single reaction vessel so as to achieve approximately equimolar yield of products. The ratios are determined as a function of the length of the amplicon and the length of other amplicons being simultaneously tested. The primers may desirably be for p53 gene sequences.

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METHODS AND COMPOSITIONS FOR MULTIPLEX AMPLIFICATION OF NUCLEIC ACIDS

RELATED APPLICATION

This application claims priority to U.S. Provisional Application, Serial No. 60/050,405, filed on June 20, 1997, the text of which is expressly incorporated herein.

BACKGROUND OF THE INVENTION

The polymerase chain reaction (PCR) is a simple and versatile method to amplify *in vitro* a specific segment of DNA for subsequent study (Saiki *et al.*, *Science 230*:1350 (1985); Saiki *et al.*, *Science 235*:487 (1985)). The PCR method has gained widespread use in biomedical research, and has revolutionized the accurate and early diagnosis of many inherited and acquired genetic disorders (Eisenstein, N. *Engl. J. Med. 322*:178 (1990)), particularly those caused by point mutations or small insertions or deletions including sickle cell anemia (Saiki *et al.*, *Science 230*:1350 (1985)), hemophilia A (Kogan *et al.*, *N. Engl. J. Med. 317*:985 (1987)), Tay-Sach's disease (Myerowitz, *Proc. Natl. Acad. Sci. USA 85*:3955 (1988); Myerowitz *et al.*, *J. Biol. Chem. 263*:18587 (1988)), cystic fibrosis (Riordan *et al.*, *Science 245*:1066 (1989)), and many others. With PCR, it is also possible to detect heterozygotic carriers in recessive disorders.

Polymerase chain reaction (PCR) is used for a variety of purposes. PCR can be used to amplify genomic DNA or other sources of nucleic acids for analysis. It is often desirable to be able to achieve equimolar yields of different length amplicons when performing multiplex PCR or multiple PCR reactions. Having an approximately equimolar yield of amplicons is particularly useful, for example, when approximately equal representation of certain regions of genomic DNA amplified after multiplex PCR is desired. Prior to the methods of present invention, finding the appropriate experimental conditions useful to achieve this result has been difficult because PCR amplifies nucleic acids having different lengths with different efficiencies. The yield of longer amplicons is often less than the yield of shorter amplicons because of those differences in PCR amplification efficiency. Figure 1 shows the difference in yields that one might expect, for example, when starting with equal primer concentrations used to amplify amplicons of varying lengths: A, B, C. There is a continuing need in the art for methods which permit the amplification of different sequences with the same efficiency so that approximately equimolar products result.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method of performing multiplex PCR which achieve approximately equimolar products.

It is another object of the invention to provide a set of primers for amplification of p53.

It is yet another object of the invention to provide a set of primers for amplification of p53 to achieve approximately equimolar products.

It is still another object of the invention to provide a mixture of primers for

performing multiplex PCR.

These and other objects of the invention are provided by one or more of the embodiments provided below. In one embodiment of the invention a method of performing multiple polymerase chain reactions in a single vessel is provided. The method comprises the steps of priming DNA synthesis on a template in a vessel with at least two sets of primers. The primers are present in the vessel at a predetermined ratio which is described by the formula:

$$C_A = C_L (L_A \div L_L)^2$$

C_A is the concentration of primers for an amplicon A. C_L is the concentration of primer for the longest amplicon. L_A is the length of the amplicon A. L_L is the length of the longest amplicon.

Another embodiment provided by the invention is a method of performing multiple polymerase chain reactions in a single vessel. The method comprises priming DNA synthesis on a genomic p53 template in a vessel with ten sets of primers which amplify exons 2-11 of p53. The primers are shown in SEQ ID NO: ID NOS: 1-20. The primers are present in the vessel at the following ratio: exon 2 (89.4): exon 3 (26.9): exon 4 (450): exon 5 (245.8): exon 6 (138.3): exon 7 (101.8): exon 8 (193.0): exon 9 (70.8): exon 10 (146.5): exon 11 (177.3).

According to still another embodiment of the invention a set of primers for performing multiple polymerase chain reactions in a single vessel is provided. The set comprises twenty primers having sequences as shown in SEQ ID NO: 1-20.

According to yet another embodiment of the invention a mixture of primers for performing multiplex polymerase chain reaction is provided. The primers are present in the mixture at a predetermined ratio to each other. The ratio of the concentrations of

the primers is described by:

$$C_A = C_L (L_A \div L_L)^2$$

wherein C_A is the concentration of primers for an amplicon A; wherein C_L is the concentration of primer for the longest amplicon; wherein L_A is the length of the amplicon A; and wherein L_L is the length of the longest amplicon.

The present invention thus provides the art with a method useful for performing multiplex PCR. This method is particularly useful for amplification of multiple exons of p53. Moreover, a particular primer set useful for performing such multiplex PCR is also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the difference in yields that one might expect, for example, when starting with equal primer concentrations used to amplify amplicons of varying lengths: A, B, C.

Figure 2 illustrates the relationship for given values X and L_L, using the amplicons from different exons of the human p53 gene as an example.

DETAILED DESCRIPTION OF THE INVENTION

It is a discovery of the present invention that approximately equimolar yields of amplicons of varying lengths can be easily produced by multiplex PCR. It has been determined that varying the primer concentrations as a function of the lengths of amplicons yields approximately equimolar amounts of amplicons of varying lengths. The relationship between primer concentration and the length of amplicons is as follows:

$$C_A = C_L(L_A / L_L)^X$$

wherein C_A = the concentration of primers for an amplicon A;

C_L = the concentration of primer for the longest amplicon;

L_A = the length of amplicon A;

L_L = the length of the longest amplicon; and

X is usually not zero and is often between one and three.

This relationship can be placed in a computer readable medium or be used with a computer system if desired.

Figure 2 illustrates the relationship for given values X and L_L, using the amplicons from different exons of the human p53 gene as an example. Using primer concentrations as set forth, for example in Figure 2, one skilled in the art can determine the optimum set of primer concentrations to yield approximately equimolar yields of varying length amplicons in a multiplex or multiple PCR. Preferably, primers having both comparable base composition and comparable melting temperatures are used. Also preferably, Mg^{+2} concentration, annealing temperatures, and cycling times of the PCR are optimized prior to choosing the desired set of primer concentrations in accordance with the present invention.

PCR techniques applicable to the present invention include *inter alia* those discussed in PCR PRIMER: A LABORATORY MANUAL, Dieffenbach, C.W. and Dveksler, G.S., eds., Cold Spring Harbor Laboratory Press (1995).

The present application further provides primer sequences, primer concentrations, and experimental conditions useful in the amplification of the coding region of the human p53 gene. Particularly useful primers for amplification of exons of the p53 gene are set forth in Table 1.

TABLE 1

p53 Primer Set

20 primers in 1 mM Tris-HC1, pH 7.4, 0.1 mM EDTA, sequences:

Exon 2:	5'-TCATGCTGGATCCCCACTTTTCCTCTTG-3'
	5'TGGCCTGCCCTTCCAATGGATCCACTCA-3'
Exon 3:	5'-AATTCATGGGACTGACTTTCTGCTCTTGTC-3'
	5'-TCCAGGTCCCAGCCCAACCCTTGTCC-3'
Exon 4:	5'-GTCCTCTGACTGCTCTTTTCACCCATCTAC-3'
	5'-GGGATACGGCCAGGCATTGAAGTCTC-3'
Exon 5:	5'-CTTGTGCCCTGACTTTCAACTCTGTCTC-3'
	5'-TGGGCAACCAGCCCTGTCGTCTCTCCA-3'
Exon 6:	5'-CCAGGCCTCTGATTCCTCACTGATTGCTC-3'
	5'-GCCACTGACAACCACCCTTAACCCCTC-3'
Exon 7:	5'-GCCTCATCTTGGGCCTGTGTTATCTCC-3'
	5'-GGCCAGTGTGCAGGGTGGCAAGTGGCTC-3'
Exon 8:	5'-GTAGGACCTGATTTCCTTACTGCCTCTTGC-3'
	5'-ATAACTGCACCCTTGGTCTCCTCCACCGC-3'
Exon 9:	5'-CACTTTTATCACCTTTCCTTGCCTCTTTCC-3'
	5'-AACTTTCCACTTGATAAGAGGTCCCAAGAC-3'
Exon 10:	5'-ACTTACTTCTCCCCCTCCTCTGTTGCTGC-3'
	5'-ATGGAATCCTATGGCTTTCCAACCTAGGAAG-3'
Exon 11:	5'-CATCTCTCCTCCCTGCTTCTGTCTCCTAC-3'
	5'-CTGACGCACACCTATTGCAAGCAAGGGTTC-3'

Table 2 shows particularly useful concentrations of the primers set forth in Table 1 for multiplex PCR amplification using the experimental conditions set forth in Table 3.

TABLE 2

Primer Concentrations in p53 Primer Set

Values of X	**2**	
Typical values of C_L	**450 nM**	

	Amplicon	Length	Primer Concs
Longest	4	**368 bp**	450.0 nM
	5	272 bp	245.8 nM
	8	241 bp	193.0 nM
	11	231 bp	177.3 nM
	10	210 bp	146.5 nM
	6	204 bp	138.3 nM
	7	175 bp	101.8 nM
	2	164 bp	89.4 nM
	9	146 bp	70.8 nM
Shortest	3	90 bp	26.9 nM

TABLE 3

Multiplex PCR

Start with 250 ng of Template DNA.

PCR Components for 100 ul PCR in 0.2 ml thin walled tubes:

	Stock Conc	Final Conc	for 1 reaction
Buffer (No Mg)	10 X	1 X	10.0 ul
$MgCl_2$	25 mM	2.5 mM	10.0 ul
dATP	10 mM	200 uM	2.0 ul
dCTP	10 mM	200 uM	2.0 ul
dGTP	10 mM	200 uM	2.0 ul
dTTP	10 mM	200 uM	2.0 ul
Taq GOLD	5 U/ul	10 U	2.0 ul
p53 Primer Set	20 X	1 X	5.0 ul
Water			
Human genomic DNA		250 ng	
		Total Volume	100.0 ul

Final Concentrations in Buffer (No Mg) are 10 mM Tris-HCl (pH 8.3), 50 mM KCl
Taq GOLD is AmpliTaq Gold™ from Perkin Elmer catalog # N808-0243

PCR Cycles:

	94 C	10 min
35 Cycles:	94 C	30 sec
	60 C	30 sec
	72 C	45 sec
	72 C	10 min

To visualize amplicons by gel Analysis:
Visualize PCR products on 4% NuSieve Agarose Gel
NuSieve™ Agarose 3:1 is from FMC catalog # 50092
Load 5 ul of PCR + loading buffer
Use 50 bp Ladder (Gibco/BRL catalog # 10416-014) as size marker
Run gel at 125 Volts for 30 min. to 90 min.

Expected PCR Products:

Amplicon	Length
Exon 2	164 bp
Exon 3	90 bp
Exon 4	368 bp
Exon 5	272 bp
Exon 6	204 bp
Exon 7	175 bp
Exon 8	241 bp
Exon 9	146 bp
Exon 10	210 bp
Exon 11	225 bp

Order in Gel:

Amplicon	Length
Exon 4	368 bp
Exon 5	272 bp
Exon 8	241 bp
Exon 11	225 bp
Exon 10	210 bp
Exon 6	204 bp
Exon 7	175 bp
Exon 2	164 bp
Exon 9	146 bp
Exon 3	90 bp

Using the methods and reagents provided herein, we achieved multiplex PCR amplification of coding regions shown of the human p53 gene in approximately equimolar amounts. That desirable result was achieved in a single-tube reaction. The achievement of such desirable results with the remarkable convenience of a single tube reaction further illustrates the contribution to the art made by the present invention.

The methods and compositions of the present invention are useful in virtually any context in which equimolar yields of various PCR products are desired. Such contexts include without limitation paternity testing, forensic analysis, genetic screening, polymorphism detection, and mutation analyses. The present invention can be used to amplify nucleic acids for all forms of sequence analysis known to those skilled in the art. Sequence analysis techniques includes, for example, dideoxy-sequencing and sequence analysis using high-density nucleic acid arrays: the GeneChip® probe arrays or VLSIPS™ technology of Affymetrix, Inc. High density nucleic acid arrays are discussed for example in Chee, M., Yang, R., Hubbell, E., Berno, A., Huang, X.C., Stern, D., Winkler, J., Lockhart, D.J., Morris, M.S.,, & Fodor, S.P., Science **5287**, 610-614 (1996), U.S. Patent No. 5,445,934, and International Publication No. WO 95/11995 corresponding to PCT Application No. PCT/US94/12305.

The p53 gene and its protein product are discussed in *Molecular Biology of the Cell,* 3rd Edition, Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D., Garland Publishing (1994) at pages 889 and 1284-1289.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of any appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

SEQUENCE LISTING

(1) GENERAL INFORMATION

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(ii) TITLE OF THE INVENTION: METHODS AND COMPOSITIONS FOR MULTIPLEX AMPLIFICATION OF NUCLEIC ACIDS

(iii) NUMBER OF SEQUENCES: 20

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(v) COMPUTER READABLE FORM:
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(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCATGCTGGA TCCCCACTTT TCCTCTTG
28

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGGCCTGCCC TTCCAATGGA TCCACTCA
28

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AATTCATGGG ACTGACTTTC TGCTCTTGTC
30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCCAGGTCCC AGCCCAACCC TTGTCC
26

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTCCTCTGAC TGCTCTTTTC ACCCATCTAC
30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGATACGGC CAGGCATTGA AGTCTC
26

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTTGTGCCCT GACTTTCAAC TCTGTCTC
28

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGGGCAACCA GCCCTGTCGT CTCTCCA
27

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCAGGCCTCT GATTCCTCAC TGATTGCTC
29

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCCACTGACA ACCACCCTTA ACCCCTC
27

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCCTCATCTT GGGCCTGTGT TATCTCC
27

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGCCAGTGTG CAGGGTGGCA AGTGGCTC
28

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTAGGACCTG ATTTCCTTAC TGCCTCTTGC
30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATAACTGCAC CCTTGGTCTC CTCCACCGC
29

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CACTTTTATC ACCTTTCCTT GCCTCTTTCC
30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AACTTTCCAC TTGATAAGAG GTCCCAAGAC
30

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ACTTACTTCT CCCCCTCCTC TGTTGCTGC

29

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ATGGAATCCT ATGGCTTTCC AACCTAGGAA G
31

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CATCTCTCCT CCCTGCTTCT GTCTCCTAC
29

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTGACGCACA CCTATTGCAA GCAAGGGTTC
30

Claims

1. A method of performing multiple polymerase chain reactions in a single vessel, comprising:

priming DNA synthesis on a template in a vessel with at least two sets of primers, wherein the primers are present in the vessel at a predetermined ratio, wherein the ratio is described by:

$$C_A = C_L (L_A \div L_L)^2$$

wherein C_A is the concentration of primers for an amplicon A; wherein C_L is the concentration of primer for the longest amplicon; wherein L_A is the length of the amplicon A; and wherein L_L is the length of the longest amplicon.

2. The method of claim 1 wherein the template is genomic DNA encoding p53.

3. The method of claim 1 wherein the template is a cDNA encoding p53.

4. The method of claim 1 wherein the primers amplify at least 2 exons of p53 selected from the group consisting of exons 2-11.

5. The method of claim 1 wherein the primers amplify at least 4 exons of p53 selected from the group consisting of exons 2-11.

6. The method of claim 1 wherein the primers amplify exons 2-11 of p53.

7. The method of claim 4 wherein the primers are selected from those shown in SEQ ID NO: ID NOS: 1-20.

8. The method of claim 5 wherein the primers are selected from those shown in SEQ ID NO: ID NOS: 1-20.

9. The method of claim 6 wherein the primers are shown in SEQ ID NO: ID NOS: 1-20.

10. The method of claim 9 wherein the primers are present in the following ratios: exon 2 (89.4): exon 3 (26.9): exon 4 (450): exon 5 (245.8): exon 6 (138.3): exon 7 (101.8): exon 8 (193.0): exon 9 (70.8): exon 10 (146.5): exon 11 (177.3).

11. A method of performing multiple polymerase chain reactions in a single vessel, comprising:

priming DNA synthesis on a genomic p53 template in a vessel with ten

sets of primers which amplify exons 2-11 of p53, wherein the primers are shown in SEQ ID NOS: 1-20, wherein the primers are present in the vessel at the following ratios: exon 2 (89.4), exon 3 (26.9), exon 4 (450), exon 5 (245.8), exon 6 (138.3), exon 7 (101.8), exon 8 (193.0), exon 9 (70.8), exon 10 (146.5), exon 11 (177.3).

12. A kit comprising a set of primers for performing multiple polymerase chain reactions in a single vessel, comprising:

twenty primers having sequences as shown in SEQ ID NO: ID NOS: 1-20.

13. The kit of claim 12 wherein the ratio of the concentrations of the primers is described by:

$$C_A = C_L (L_A \div L_L)^2$$

wherein C_A is the concentration of primers for an amplicon A; wherein C_L is the concentration of primer for the longest amplicon; wherein L_A is the length of the amplicon A; and wherein L_L is the length of the longest amplicon.

14. The kit of claim 12 wherein the ratio of the primers is: exon 2 (89.4): exon 3 (26.9): exon 4 (450): exon 5 (245.8): exon 6 (138.3): exon 7 (101.8): exon 8 (193.0): exon 9 (70.8): exon 10 (146.5): exon 11 (177.3).

15. A mixture of primers for performing multiplex polymerase chain reaction, wherein the primers are present in the mixture at a predetermined ratio to each other, wherein the ratio of the concentrations of the primers is described by:

$$C_A = C_L (L_A \div L_L)^2$$

wherein C_A is the concentration of primers for an amplicon A; wherein C_L is the concentration of primer for the longest amplicon; wherein L_A is the length of the amplicon A; and wherein L_L is the length of the longest amplicon.

16. The mixture of claim 15 which comprises at least 4 primers.

17. The mixture of claim 15 which comprises at least 6 primers.

18. The mixture of claim 15 which comprises at least 8 primers.

19. The mixture of claim 15 which comprises at least 10 primers.

20. The mixture of claim 15 which comprises at least 12 primers.

21. The mixture of claim 15 which comprises at least 14 primers.

22. The mixture of claim 15 which comprises at least 16 primers.

23. The mixture of claim 15 which comprises at least 18 primers.

24. The mixture of claim 15 which comprises at least 20 primers.

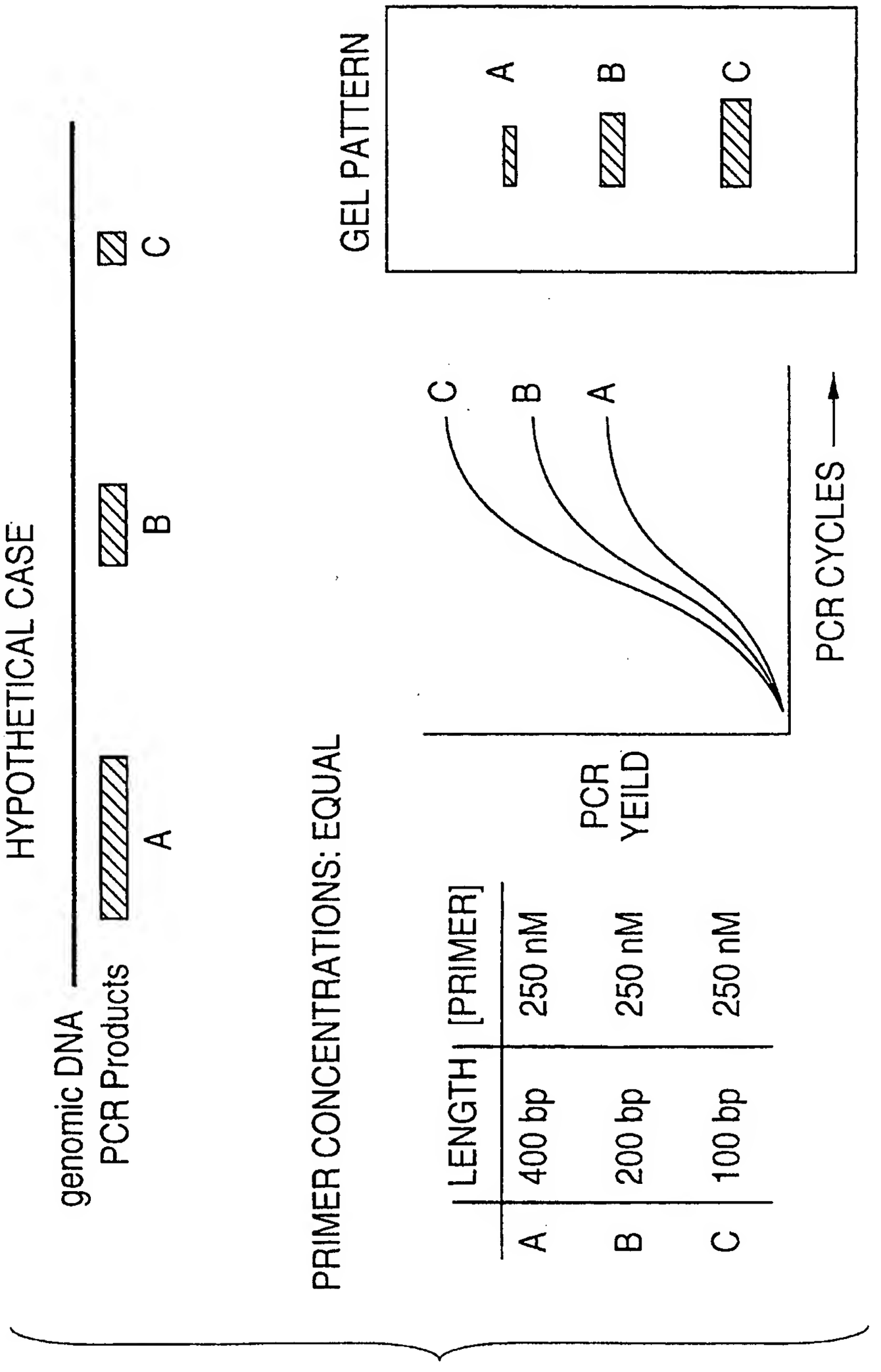

HYPOTHETICAL CASE
genomic DNA
PCR Products
A
B
C
PRIMER CONCENTRATIONS: EQUAL
LENGTH
[PRIMER]
A
400 bp
250 nM
B
200 bp
250 nM
C
100 bp
250 nM
PCR YEILD
PCR CYCLES
GEL PATTERN
A
B
C

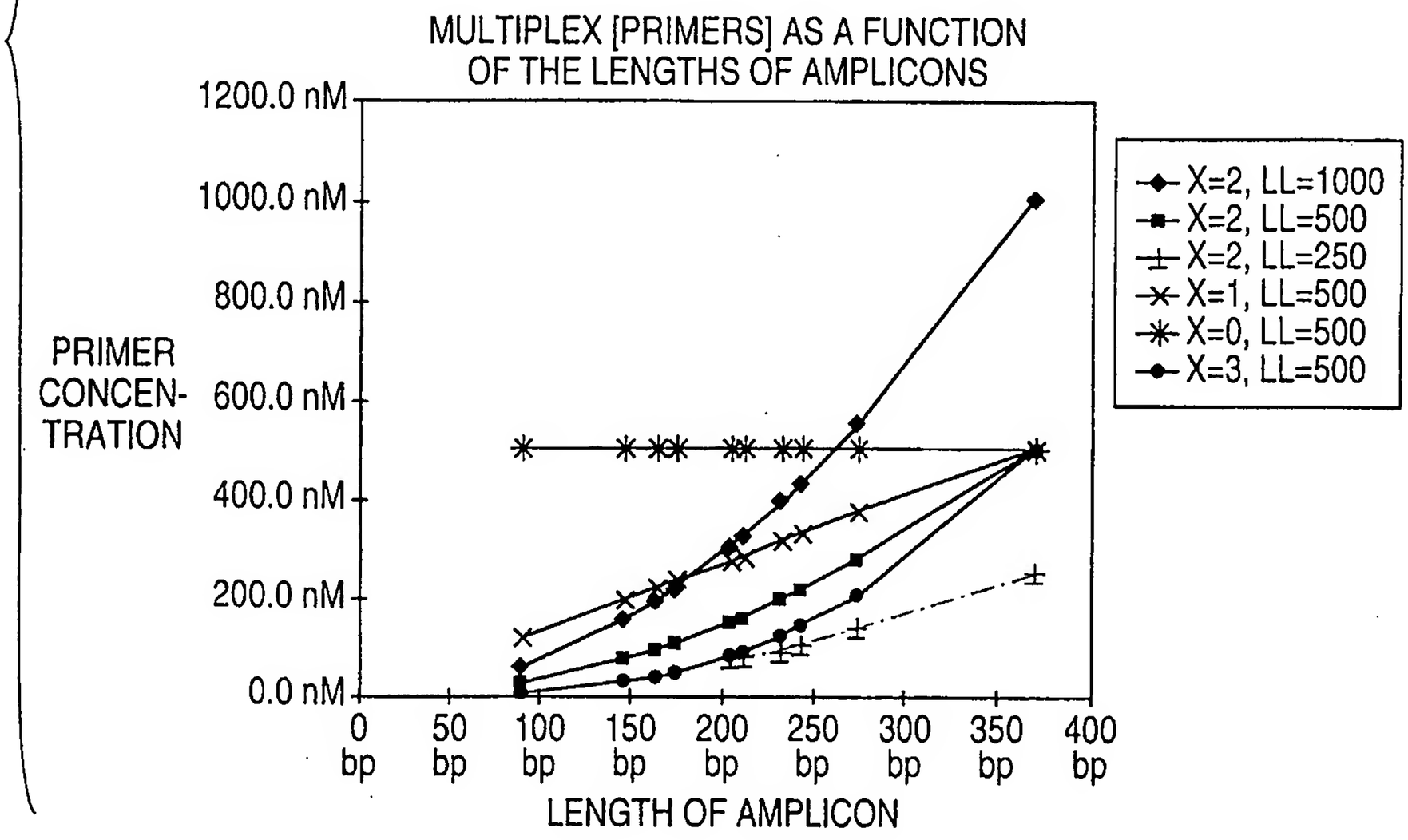
FIG. 1

FIG. 2

MULTIPLEX PCR PRIMER CONCENTRATIONS AS A FUNCTION OF THE LENGTHS OF AMPLICONS

VALUES OF X	2	2	2	1	0	3
TYPICAL VALUES OF L_L	1000 nM	500 nM	250 nM	500 nM	500 nM	500 nM

	AMPLI-CON	LENGTH	X=2, L_L=1000	X=2, L_L=500	X=2, L_L=250	X=1, L_L=500	X=0, L_L=500	X=3, L_L=500
LONGEST	4	368 bp	1000.0 nM	500.0 nM	250.0 nM	500.0 nM	500.0 nM	500.0 nM
	5	272 bp	546.3 nM	273.2 nM	136.6 nM	369.6 nM	500.0 nM	201.9 nM
	8	241 bp	428.9 nM	214.4 nM	107.2 nM	327.4 nM	500.0 nM	140.4 nM
	11	231 bp	394.0 nM	197.0 nM	98.5 nM	313.9 nM	500.0 nM	123.7 nM
	10	210 bp	325.6 nM	162.8 nM	81.4 nM	285.3 nM	500.0 nM	92.9 nM
	6	204 bp	307.3 nM	153.7 nM	76.8 nM	277.2 nM	500.0 nM	85.2 nM
	7	175 bp	226.1 nM	113.1 nM	56.5 nM	237.8 nM	500.0 nM	53.8 nM
	2	164 bp	198.6 nM	99.3 nM	49.7 nM	222.8 nM	500.0 nM	44.3 nM
	9	146 bp	157.4 nM	78.7 nM	39.4 nM	198.4 nM	500.0 nM	31.2 nM
SHORTEST	3	90 bp	59.8 nM	29.9 nM	15.0 nM	122.3 nM	500.0 nM	7.3 nM



INTERNATIONAL SEARCH REPORT

Int ıtional Application No
PCT/US 98/12779

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 01909 A (VISIBLE GENETICS INC ;DIAMANDIS ELEFTHERIOS (CA); DUNN JAMES M (CA) 25 January 1996 see page 11, line 1 - page 14, line 6 see page 19 - page 29 see claims 16-33 ---	1-24
A	WO 96 10648 A (PROMEGA CORP) 11 April 1996 see page 9, line 23 - page 35 ---	1-24
A	WO 96 39535 A (VIJG JAN ;LI DAIZONG (US)) 12 December 1996 see page 7, line 15 - page 10, line 14 ---	1-24
A	EP 0 648 845 A (EASTMAN KODAK CO) 19 April 1995 see page 2, line 49 - page 3, line 2 see page 5, line 37 - line 47 --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 October 1998	06/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

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INTERNATIONAL SEARCH REPORT

Int· :ional Application No
PCT/US 98/12779

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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